PEPTIDE EPITOPES RECOGNIZED BY ANTI-FILAGGRIN AUTO-ANTIBODIES IN SERUM FROM RHEUMATOID ARTHRITIS PATIENTS

The present invention relates to novel preparations of antigens which are specifically recognized by rheumatoid arthritis-specific autoantibodies.

Rheumatoid arthritis (hereafter abbreviated to "RA") is the most frequent of the chronic inflammatory rheumatisms. It is an autoimmune disease, and the serum from affected patients contains autoantibodies, certain of which are specific, and may constitute a marker for this disease, allowing its diagnosis even at early stages. Research has thus been carried out with a view to identifying antigens recognized by these antibodies, in order to obtain purified preparations thereof which can be used in conventional techniques of immunological diagnosis.

Autoantibodies which are specifically present in RA patients, and which react with a rat oesophagial epithelial antigen, were described for the first time by B.J.J. Young et al. in Br. Med. J. 2:97-99, (1979). These autoantibodies were, at the time, named "anti-keratin antibodies".

During previous work, the inventors' team obtained, from human and murine malpighian epithelia, preparations of antigens related to filaggrin and to profilaggrin which are recognized specifically by antibodies present in serum from rheumatoid arthritis patients, and showed that the "anti-keratin antibodies" were in fact anti-filaggrin autoantibodies (hereafter named "AFA"). Application EP 0 511 116 describes these antigenic preparations and their use for diagnosing rheumatoid arthritis.

Filaggrins are a family of proteins which have been identified in various species, inter alia in humans, rats, mice and guinea pigs, in keratinizing malpighian epithelia [for a review on filaggrins, cf. Dale et al. [The Keratinocyte Handbook, Cambridge

University Press, pp. 323-350, (1994)]]. They are derived from the dephosphorylation and proteolysis of a precursor, profilaggrin, which consists essentially of repeated filaggrin domains separated by interdomain peptide segments.

The gene encoding profilaggrin is composed of repeated subunits, each of which encodes a filaggrin molecule, separated by portions encoding the inter-domain peptide segments. All the repeat units encoding each of the human filaggrins have the same length (972 base pairs in humans); however, in humans, considerable sequence variation (10-15%) is observed from one subunit to the other. While most are conservative, some of these variations induce amino acid changes and, in certain cases, changes in the electrical charge of the protein. Thus, human filaggrins form, independently of post-transcriptional modifications, a heterogeneous population of molecules of similar size, but of different sequences and charges (pHi equal to 8.3 ± 1.1) [Gan et al., Biochem. 29, pp. 9432-9440 (1990)].

Profilaggrin is a protein of high molecular weight (approximately 400,000 in humans) which is soluble in the presence of high concentrations of salts or of urea. It possesses a high content of basic amino acids (arginine and histidine) and of glycine, serine and glutamic acid. It is low in nonpolar amino acids, and contains neither methionine, nor cysteine, nor tryptophan. It is highly phosphorylated on serine residues, which gives it an isoelectric point close to neutrality.

Profilaggrin is cleaved into filaggrin units during a complex maturation process which involves dephosphorylation, followed by cleavage by proteases at the interdomain segments. This cleavage generates, first of all, fragments of intermediate size, and then the functional filaggrin molecules.

Filaggrins derived from the dephosphorylation and cleavage of profilaggrin are basic proteins, the amino acid content of which is similar to that of

profilaggrins. They participate in the organization of keratin filaments, and undergo a progressive maturation during which the arginine residues, which are basic, are converted into citrulline residues, which are neutral, under the action of peptidylarginine deiminase [Harding C.R. and Scott I.R., J. Mol. Biol. 170, pp. 651-673 (1983)]. This leads to a reduction in their affinity for keratins, from which they detach; they are then totally degraded under the action of various proteases.

Filaggrin and profilaggrin properties have been particularly well studied in rats, in mice and in humans. The size of profilaggrin varies, according to the species, from 300 to 400 kD, and that of filaggrins from 27 to 64 kD.

The polymorphism observed in humans between the sequences of filaggrin units within the same profilaggrin gene does not appear in rats and mice. Filaggrins also exhibit great inter- and intraspecific variability in their sequence. This variability does not however affect their functional properties, or their overall amino acid composition, or their biochemical properties. Similarly, the tissue localizations of profilaggrin and of filaggrins are identical in the various mammals studied.

In continuing their work, the inventors noticed that profilaggrin present in keratohyalin granules of human epidermis was not recognized, unlike filaggrins, by AFAs [Simon et al. Clin. Exp. Immunol. 100, 90-98 (1995)]. They then tested the reactivity of AFAs with recombinant filaggrin, and observed that this was not recognized either. Conversely, it had previously been observed that the forms of human epidermal filaggrin principally recognized by AFAs were the acido-neutral forms described by Simon et al. [J. Clin. Invest., 92, 1387, (1993)], and in Application EP 0 511 116. The fact that these acido-neutral forms correspond to a late maturation stage of filaggrin made it possible to presume that all or part of the post-translational

modifications which intervene up to this stage are involved in the formation of the epitopes recognized by AFAs.

To verify this hypothesis, the inventors attempted to reproduce these post-translational modifications *in vitro*, using recombinant filaggrin, in order to determine which are capable of influencing filaggrin antigenicity.

They thus in fact observed that citrullination of filaggrin was enough to generate epitopes recognized by AFAs. Specifically, they observed, by carrying out *in vitro* deimination of recombinant filaggrin, that replacing at least one portion of the arginines with citrullines allows the production of an antigen which is recognized specifically by AFAs present in serum from RA patients. They also located regions which, after citrullination, were highly immunoreactive with respect to anti-filaggrin autoantibodies. They are in particular the region corresponding to the C-terminal portion (amino acids 144 to 324), and in particular to amino acids 144 to 314, as well as the region corresponding to amino acids 76 to 144 and the region corresponding to amino acids 71 to 119, of a human filaggrin unit. This work resulted in the production of artificial antigens, which are recognized specifically by AFAs present in serum from RA patients, and which consist of recombinant or synthetic polypeptides derived from the sequence of filaggrin or from portions of it, by substituting at least one arginine residue with a citrulline residue. These antigens, as well as their use, form the subject of Application FR 96/10651, filed on 30 August 1996 in the name of Biomérieux.

In continuing their work, the inventors managed to select, using the sequence of one filaggrin unit, peptides in which substituting at least one arginine residue with a citrulline residue gives rise to epitopes which are recognized specifically by AFAs present in serum from RA patients.

The sequences of these peptides are identified

in the attached sequence listing under the numbers SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 6.

"Filaggrin unit" is intended to mean a polypeptide, the sequence of which is that of the translation product of any one of the subunits encoding a filaggrin domain of the human, or any other species, profilaggrin gene, or is a consensus sequence, this theoretical sequence being obtained from filaggrin domain sequences.

The inventors have now identified epitopes recognized by anti-filaggrin autoantibodies: these epitopes comprise a tripeptide motif centred on a citrulline residue, which is specifically present on the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6, and which is absent from the sequence SEQ ID NO: 4.

It is in particular the tripeptide motif Ser-Cit-His in which Cit represents a citrulline residue.

A subject of the present invention is a peptide which constitutes an epitope recognized by anti-filaggrin autoantibodies present in serum from rheumatoid arthritis patients, characterized in that said epitope comprises a tripeptide motif centred on a citrulline residue, which is specifically present on at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6.

According to a preferred embodiment of the present invention, said peptide comprises at least one pentapeptide motif centred on a citrulline residue, which is present on at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6.

Advantageously, said peptide comprises the tripeptide motif Ser-Cit-His in which Cit represents a citrulline residue.

By way of example, mention will be made of peptides derived, by citrullination, from peptides

which comprise the pentapeptide motif X1-Ser-Arg-His-X2 in which X1 = Ser or Gly, and X2 = Ser or Pro, and among them, peptides which comprise the hexapeptide motif X0-X1-Ser-Arg-His-X2 or the heptapeptide motif X0-X1-Ser-Arg-His-X2-X3, in which X1 and X2 are as defined above, X0 = Asp and X3 = Gly or Arg.

The peptides in accordance with the invention allow the preparation of artificial antigens which are recognized specifically by anti-filaggrin autoantibodies present in serum from rheumatoid arthritis patients. These artificial antigens also form part of the subject of the present invention.

Artificial antigens in accordance with the invention comprise at least one peptide epitope centred on a citrulline residue as defined above. They consist for example of peptides of at least 5 amino acids, preferably at least 10 amino acids, and advantageously at least 14 amino acids. They can be peptides consisting of at least one fragment of at least one of the citrullinated peptides derived from the sequences SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 6, or containing at least one such fragment. These peptides can comprise several citrullinated epitopes which are specifically recognized by AFAs, and which are of identical or different sequences.

The term "peptide" as used in the present application means in particular protein or protein fragment, or oligopeptide, which is extracted, separated or substantially isolated or synthesized, in particular those obtained by chemical synthesis or by expression in a recombinant organism; any peptide in the sequence of which one or more amino acids of the L series are replaced with an amino acid of the D series, and vice versa; any peptide in which one at least of the CO-NH bonds, and advantageously all the CO-NH bonds, of the peptide chain is (are) replaced with one or more NH-CO bonds; any peptide in which one at least of the CO-NH bonds, and advantageously all the CO-NH bonds, is or are replaced with one or more NH-CO

bonds, the chirality of each aminoacyl residue, whether or not it is involved in one or more abovementioned CO-NH bonds, being either conserved or inverted with respect to the aminoacyl residues constituting a reference peptide, these compounds also being referred to as immunoretroids, a mimotope, etc.

Antigens in accordance with the invention can for example be obtained by reacting PAD (peptidyl-arginine deiminase) with natural, recombinant or synthetic proteins or peptides comprising arginine residues and in particular comprising at least one arginine residue constituting the centre of a tri-peptide motif identical to those present in the sequences SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 6; they can also be obtained by peptide synthesis, by directly incorporating one or more citrulline residues, and preferably one or more epitopes comprising a citrulline residue as defined above, into the synthesized peptide.

A subject of the present invention is also the use of the antigens in accordance with the invention as defined above for diagnosing RA *in vitro*.

The present invention encompasses in particular antigenic compositions for diagnosing the presence of RA-specific autoantibodies in a biological sample, these compositions being characterized in that they contain at least one antigen in accordance with the invention which is optionally labelled with and/or conjugated to a carrier molecule.

A subject of the present invention is also a method for detecting RA-specific class G autoantibodies in a biological sample, this method being characterized in that it comprises:

- bringing said biological sample into contact with at least one antigen in accordance with the invention as defined above under conditions which allow the formation of an antigen/antibody complex with the RA-specific autoantibodies possibly present;
- detecting, by any suitable means, the antigen/anti-

body complex possibly formed.

This detection method can be implemented using a kit comprising at least one antigen according to the invention, as well as suitable buffers and reagents for constituting a reaction medium which allows the formation of an antigen/antibody complex, and/or means for detecting said antigen/antibody complex.

Said kit can also comprise, where appropriate, reference samples such as one or more negative serum (sera) and one or more positive serum (sera).

The present invention will be better understood with the aid of the further description which will follow, which refers to examples of preparation and of use of antigens in accordance with the invention.

EXAMPLE 1: *IN VITRO* DEIMINATION OF RECOMBINANT FILAGGRIN WITH PEPTIDYLARGININE DEIMINASE (P.A.D.)

Recombinant filaggrin is produced according to the following protocol:

A DNA fragment encoding a filaggrin unit is amplified from human genomic DNA (RAJI cells: ATCC CCL86) by PCR using the 2 primers below:

5' primer:

5' TTCCTATACCAGGTGAGCACTCAT 3'

3' primer:

5' AGACCCTGAACGTCCAGACCGTCCC 3'

The amplification product is cloned into the SmaI site of the vector pUC19. The recombinant clones are selected by verifying the presence of a 972-bp insert obtained after digestion with SacI and XbaI. This insert is then subcloned into pUC19. The insert resulting from this subcloning is then transferred into the vector pGEX (sold by the company Pharmacia), between the EcoRI and HindIII sites. In *E. coli*, the expression vector thus obtained expresses filaggrin in fusion with glutathione-S-transferase (GST) under the control of the Tac prokaryotic promoter. Recombinant protein synthesis is induced by adding isopropyl-β-D-galactoside (IPTG) to the culture.

The recombinant filaggrin thus obtained will be named hereafter: "fil-gst".

After electrophoresis, the existence of 9 fragments is observed, which result from a post-translational proteolysis of whole filaggrin.

The mixture of the 9 fragments is subjected to an *in vitro* deimination with peptidylarginine deiminase.

A preparation of rabbit muscle peptidylarginine deiminase (681 U/ml), sold by Takara Biomed Europe, is used according to the protocol recommended by the manufacturer.

The procedure conditions are as follows:

- reaction medium: 0.1 M Tris-HCl, 10 mM $CaCl_2$, 5 mM DTT, pH 7.4;
- enzyme/substrate ratio: 140 mU/µmol of filaggrin containing 10% of arginine, i.e. 4 mU/µmol of arginine;
- incubation: between 0 and 60 min at 50°C;
- termination of the reaction: heating for 3 min in Laemmli buffer.

The 8 reactions below are carried out in parallel.

(1) BSA (bovine serum albumin) incubated in reaction medium (1 h, 50°C) without P.A.D.

(2) BSA incubated in reaction medium (1 h, 50°C) with 60 mU of P.A.D.

(3) fil-gst incubated in reaction medium (1 h, 50°C) without P.A.D.

(4) fil-gst incubated in reaction medium (5 minutes at 50°C) with 60 mU of P.A.D.

(5) fil-gst incubated in reaction medium (15 minutes at 50°C) with 60 mU of P.A.D.

(6) fil-gst incubated in reaction medium (30 minutes at 50°C) with 60 mU of P.A.D.

(7) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of P.A.D.

(8) fil-gst incubated in reaction medium (1 h at 50°C) with 60 mU of P.A.D. and in the presence of

10 mM of N-ethylmaleimide (inhibitor of P.A.D.).

1 µl of each sample is loaded on an electrophoresis gel (12.5% SDS-PHAST® gel, Pharmacia), and electrophoresis is carried out using the PHAST-SYSTEM® apparatus (Pharmacia) under the conditions recommended by the manufacturer. After transfer onto nitrocellulose, detection is carried out either with a pool of 5 sera from RA patients, diluted to 1/2000, or with the anti-filaggrin monoclonal antibody AHF2 [Simon et al. J. Invest. Dermatol. 105, 432, (1995)] at the concentration of 0.2 µg/ml.

The antigen/antibody complex is detected by the ECL technique using a peroxidase-coupled secondary antibody.

The results show that, in the absence of citrullination reaction, the fil-gst is not recognized by sera from RA patients, whereas right from 5 minutes of citrullination, it is detected by these sera. An increase in the reactivity with the pool of sera is observed when P.A.D. is reacted for 60 minutes at 50°C.

In addition, these results make it possible to presume that one or more epitopes of high affinity exist in the COOH-terminal moiety of filaggrin (positions 144 to 324), this or these epitope(s) being repeated between positions 76 and 144.

EXAMPLE 2: CITRULLINATION OF PEPTIDES S-47-S AND S-35-R WITH P.A.D., AND CITRULLINATED PEPTIDE REACTIVITY ASSAY

The 49-amino acid peptide S-47-S of sequence (1-letter code):

NH_2-STGHSGSQHSHTTTQGRSDASRGSSGSRSTSRETRDQEQSGDGSRHSGS-COOH

corresponding to amino acids 71 to 119 of the sequence of a human filaggrin unit and comprising 6 arginine residues, and

the 37-amino acid peptide S-35-R of sequence (1-letter code):

NH_2-SQDRDSQAQSEDSERRSASASRNHRGSAQEQSRDGSR-COOH

corresponding to amino acids 155 to 191 of the sequence of a human filaggrin unit and comprising 7

arginine residues were prepared by peptide synthesis. The peptides S-47-S and S-35-R are represented in the attached sequence listing under the respective numbers SEQ ID No: 3 and SEQ ID No: 4.

These 2 peptides, as well as fil-gst, were citrullinated by reacting P.A.D. for 30 minutes at 50°C in the same reaction medium as that indicated in Example 1. The conditions specific for each peptide and for fil-gst are as follows:

- peptide S-47-S: 4 mU/µmol arginine
- peptide S-35-R: 2.7 mU/µmol arginine
- fil-gst: as indicated in Example 1.

The reactivity of each peptide and that of fil-gst, before and after reacting the enzyme, with respect to the monoclonal antibody AHF4 and to the serum from an RA patient are compared by "dot-blot".

The procedure conditions are as follows:

- 0.5 µg per deposit of each antigen (peptides, fil-gst, acido-neutral variants of filaggrin (AVF)).
- Treatment of the nitrocellulose for 45 minutes at 80°C, before immunodetection.
- RA serum used at the dilution of 1/2000; monoclonal antibody AHF4 used at a concentration of 0.2 µg/ml.

The results show that:

- AHF4 recognizes citrullinated or noncitrullinated peptide S-47-S and fil-gst, but does not recognize citrullinated or noncitrullinated S-35-R.
- S-47-S is recognized, after citrullination, by the serum of the RA patient, whereas neither citrullinated nor noncitrullinated S-35-R is recognized. The same serum recognizes moreover citrullinated fil-gst and AVFs, but does not recognize noncitrullinated fil-gst.

SEQ ID No:3

SEQ ID No:4 discrepancies.

EXAMPLE 3: SYNTHESIS OF CITRULLINATED AND NON-CITRULLINATED PEPTIDES E-12-H AND E-12-D, AND PEPTIDE REACTIVITY ASSAY

The peptides E-12-H and E-12-D were determined by reference to the nucleotide sequences of the human profilaggrin gene described by Gan S.Q. et al. [Biochemistry, 29: 9432-9440, (1990)].

The 14-amino acid peptide E-12-H of sequence (1-letter code):

NH_2-EQSADSSRHSGSGH-COOH

comprises 1 arginine residue, and

the 14-amino acid peptide E-12-D of sequence (1-letter code):

NH_2-ESSRDGSRHPRSHD-COOH

comprises 3 arginine residues.

The peptides E-12-H and E-12-D are represented in the attached sequence listing under the respective numbers SEQ ID No: 5 and SEQ ID No: 6.

These peptides were prepared by solid phase peptide synthesis.

The citrullinated peptides E-12-H and E-12-D were synthesized directly by incorporating a citrulline as a replacement for an arginine.

For the peptide E-12-D, only the arginine residue corresponding to the 8^{th} amino acid of the sequence was replaced with a citrulline during the peptide synthesis.

The reactivity of each citrullinated and non-citrullinated peptide was assayed with respect to a normal serum, to two sera from RA patients, to anti-filaggrin antibodies (AFAs) purified from a pool of 45 sera from RA patients and to anti-filaggrin antibodies purified from 12 sera from RA patients, respectively.

EXPERIMENTAL PROTOCOL:

The wells of Nunc Maxisorp microtitration plates were covered using the noncitrullinated and citrullinated peptides E-12-D and E-12-H, respectively,

diluted to a concentration of 5 µg/ml in a PBS buffer (pH: 7.4) and incubated overnight at 4°C (final volume: 100 µl/well). The wells were saturated for 30 minutes at 37°C with 200 µl/well of PBS-Tween 20 (0.05%), 2.5% gelatin. The negative control serum (normal serum) was diluted to 1/120. The anti-filaggrin antibodies were diluted in PBS-Tween 20 (0.05%)-gelatin (0.5%) (PBS TG) such that the final concentrations of anti-filaggrin autoantibodies are those indicated in the attached Table I. The negative control serum, the RA sera and the anti-filaggrin antibodies were added (final volume: 100 µl/well) and subjected to incubation for 1 hour at 37°C and overnight at 4°C. Peroxidase-labelled goat anti-human immunoglobulin gamma heavy chain antibodies (sold by the company Southern Biotechnologies) were added to each well (dilution in PBSTG: 1/2000, final volume: 100 µl/well) and subjected to incubation for 1 hour at 37°C. The revelation was carried out by adding ortho-phenylenediamine (2 mg/ml, for 10 minutes).

The results presented in the attached Table I are given as a ratio of OD at 492 nm: citrullinated peptide signal/noncitrullinated peptide signal.

These results show that, in the majority of cases, the citrullinated peptide/noncitrullinated peptide OD ratio is higher than 1, and thus illustrate the good sensitivity of the citrullinated peptides, compared to the noncitrullinated peptides, as regards their reactivity with respect to anti-filaggrin autoantibodies.

TABLE I

Peptide	Control serum	RA serum 1		RA serum 2			AFA pool			AFAs purified from 12 RA sera											
		10*	20*	5*	10*	20*	5*	10*	20*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*	10*
E-12-D	1.076	1.42	1.85	2.42	3.77	5.57	1.77	1.63	1.48	1.99	1.38	2.48	1.19	1.12	3.50	1.87	5.19	1.13	1.57	1.11	1.65
E-12-H	1	1.32	1.20	10.44	11.51	8.38	2.45	2.42	1.82	7.16	2.05	1.06	1.18	0.76	13.57	4.14	3.18	1.14	3.66	1.22	5.84

*: AFA concentration in µg/ml.